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Identification of Synaptophysin-Like Immunoreactivity in the Sea Anemone *Condylactis gigantea* (Cnidaria: Anthozoa)

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Abstract. Synaptophysin is a membrane protein of synaptic vesicles that serves as an antigenic marker for nervous and endocrine systems in mammals. Monoclonal antisera generated against synaptophysin were used for immunocytochemical staining in tissues of the tentacles of the sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Specific staining, visible at the light and electron microscope levels, was found in the tentacle. Proteins were extracted from the tissues and solubilized. Using SDS-polyacrylamide gel electrophoresis and Western blotting, we identified proteins with apparent molecular weights of 38,000, 78,000, and 114,000. The data suggest the tissues of this anthozoan contain synaptophysin-like proteins with molecular properties similar to those of mammalian neurons.

Introduction

Neural communication is accomplished at the synapse, a highly specialized structure where a nerve cell comes into apposition with its target. Synaptic vesicles, multimolecular neurotransmitter "packets" within neurons, are well-known storage compartments that sequester peptide and nonpeptide transmitter molecules used during the intercellular signaling process (Kelly, 1993). The release of neurotransmitter is a highly regulated, plastic event that can be fine-tuned by the neuron through a series of changes in intracellular levels of seconds messengers and related protein phosphorylation events. The modulatory effects take place at many sites including the pre- and postsynaptic terminals and the synaptic vesicles (Kelly, 1993). In the last few years progress has been made in investigating the molecular composition of mammalian synaptic vesicles and, in particular, in elucidating their membrane composition. Results suggest vesicles contain highly specific groups of abundant membrane proteins that participate in transmitter release. These groups include proteins thought to control the mobilization of vesicles (Sudhof *et al.*, 1989; De Camilli *et al.*, 1990), those involved in intracellular "trafficking" and docking (Schiavo *et al.*, 1992), and those involved in the formation of fusion pores between secretory vesicles and the plasma membrane (Jessell and Kandel, 1993).

Synaptophysin (p38) is a major integral membrane protein found in clear, small synaptic vesicles (SSVs) present in the mammalian spinal cord and retina, at neuromuscular junctions, and in the adrenal medulla (Wiedenmann and Franke, 1985). The protein, thought to be involved in the formation of transient fusion pores, shares structural features with gap junction channels (Buckley *et al.*, 1987; Thomas *et al.*, 1988; Sudhof and Jahn, 1991). Also known as synaptophysin I, one of two isoforms abundant in the brain, it represents up to 0.3% of the total protein in the cerebral cortex or 7% of synaptic vesicle protein (Knaus *et al.*, 1986; Jahn *et al.*, 1987; Wiedenmann and Franke, 1985). Synaptophysin has been isolated from several sources, sequenced, and found to be highly conserved (Leube *et al.*, 1987; Sudhof *et al.*, 1987; Johnston *et al.*, 1989; Cowan *et al.*, 1990).

Examination of neural transmission and neuroendocrine function in some lower organisms has not kept pace with that in more advanced organisms. The simplest metazoans known to possess a nervous system are those of

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the phylum Cnidaria whose members include the familiar hydra, jellyfish, sea anemones, and corals. The nervous system in these organisms has been described functionally and structurally as a diffuse nerve net (Bullock and Horridge, 1965). Yet some studies have shown condensation of neural components that form "giant axons" or a neural plexus (Mackie, 1973, 1990; Van Marle, 1977; Spencer and Arkett, 1984). Elements of these nervous systems are known to include defined sensory cells and motor cells and their processes. The molecular structure of the neural elements, however, is largely unknown. Our work involves the use of immunocytochemical techniques, as well as protein isolation and light and electron microscopy, to study protein components of the nervous system in the subtropical sea anemone *Condylactis gigantea* (Cnidaria: Anthozoa). Results of our studies suggest that the tissues of this anthozoan contain proteins with synaptophysin-like molecular properties similar to those found in the nervous and endocrine systems of mammals.

Materials and Methods

Animals

Specimens of *C. gigantea* were obtained from a supplier in Los Angeles, California, and were kept in aquaria in aerated, filtered, recirculating natural seawater (24°C) obtained from the Catalina Marine Science Center (Santa Catalina Island, California). Animals acquired from local suppliers in Philadelphia, Pennsylvania, were kept in aquaria in aerated, filtered artificial seawater and were used within a few days of purchase. Specimens were maintained on a 12/12 photoperiod and fed a diet of *Artemia* nauplii, small pieces of squid, or both three times a week. Water was changed at 2-week intervals.

Anesthetic and tentacle removal

Animals were anesthetized by immersion in seawater containing succinylcholine chloride (67 μg/ml, Sigma Chemical Co., St. Louis, Missouri). Relaxed tentacles of the anesthetized animals were about the length of normal tentacles, but somewhat smaller in diameter. Tentacles were clamped with a hemostat at the junction of the oral disk and excised proximal to the clamp.

Light microscopic immunocytochemistry

Excised tentacles were placed in 2% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 *M* sodium phosphate buffer, 0.9% NaCl), pH 6.5, for 2 h at room temperature before postfixation for 8 h at 4°C in 2% paraformaldehyde in PBS, pH 11 (Berod *et al.*, 1981). Samples were rinsed three times in PBS, pH 7.4, prior to cryoprotection by immersion for 12–24 h each in 10%, 20%, and 30% sucrose in PBS, pH 7.4, at 4°C. Tissue was placed in Tissue-Tek (Miles Inc., Elkhart, Indiana) and frozen in isopentane at liquid nitrogen temperatures. Twenty-micrometer sections were made on a Microm 500 M Cryostat (Zeiss Inc., Thornwood, New York) at −20°C and thaw-mounted on slides. Endogenous peroxidases were inactivated in 0.3% hydrogen peroxide in PBS for 20 min after a rinse in PBS for 15 min. After three successive 15-min rinses in PBS and a 1-h immersion in PBS containing 0.3% Triton X-100 and 10% normal serum (Vector Laboratories, Burlingame, California), all specimens were incubated at 4°C in mouse monoclonal primary antisera directed against (1) synaptophysin (SY38; Boehringer Mannheim Corp., Indianapolis, Indiana) at a 1:5 dilution of stock (10 μg/ml) for 48 h; or (2) synaptophysin (C7.2; Jahn *et al.*, 1985) at a 1:10,000 dilution of stock for 24 h. Three successive 15-min rinses in PBS were followed by incubation with a biotinylated goat–anti-mouse immunoglobulin, several PBS rinses, and incubation in an avidin–horseradish peroxidase complex (Vector Laboratories, Burlingame, California) following manufacturer's instructions. Tissues were incubated in a chromagen solution composed of equal volumes of 0.1% hydrogen peroxide and 0.5% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, Missouri) in PBS.

Control procedures for immunostaining included replacement of the primary antibody with goat normal serum and deletion of the secondary antibody.

SDS-PAGE and Western blotting

To demonstrate the existence of synaptophysin in the sea anemone *C. gigantea*, we isolated the constituent proteins. To minimize protein contamination, food was withheld from anemones for 3 days before extraction. Whole anemones were cleaned of debris and weighed. Specimens were chilled for 15 min at −70°C before being quickly chopped into 1-cm^3 pieces and placed in SEDTA (20 m*M* Na-ACES buffer, 0.3 *M* sucrose, 2 m*M* EDTA, pH 7.4) for 15 min with intermittent gentle hand agitation to remove mucus. Minced tissue was transferred and homogenized in TME medium [10 m*M* tris(hydroxymethyl)-aminomethane (Tris) HCl, 3 m*M* $MgCl_2$, 2 m*M* K_2-ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 8.2] in the presence of protease inhibitors (10 μg/ml leupeptin, 76.8 n*M* aprotinin, 0.7 μ*M* pepstatin, 0.83 m*M* benzamide, 0.23 m*M* phenylmethanesulfonyl fluoride, and 1 m*M* iodoacetamide) at 4°C. The homogenate was centrifuged at 800 × *g* (SS34 rotor) for 10 min at 0°C to remove insoluble debris. The supernatant was transferred and centrifuged at 9000 × *g* for

2 h at 0°C. The supernatant was reserved and stored at −70°C. Pelleted material was resuspended in TME containing 2% SDS, briefly homogenized, and centrifuged at 9000 × *g* for 2 h at 0°C. The supernatant was either used immediately or stored at −70°C. Protein content was determined by the method of Bradford (1976). Control procedures consisted of extraction of rat brain as described above.

Proteins were electrophoresed in 12.5% SDS-polyacrylamide gels by the method of Laemmli (1970) and transferred to nitrocellulose sheets. Western blotting was performed by the method of Towbin *et al.* (1979). In brief, blots were processed through incubations with blocking solution (5% skim milk in PBS) for 12 h at 4°C, then immunostained with one of the following at 4°C with gentle agitation: (1) monoclonal antibodies raised against synaptophysin (SY38; Boehringer Mannheim Corp., Indianapolis, Indiana) for 1 h at a 1:150 dilution; (2) monoclonal anti-synaptophysin (C7.2) for 3 h at a 1:2000 dilution; (3) rabbit polyclonal anti-synaptophysin (G95) for 3 h at a 1:1000 dilution; or (4) rabbit polyclonal anti-p29 for 5 h at a 1:1000 dilution. Incubations were followed by biotinylated goat-anti-mouse or goat–anti-rabbit immunoglobulin, several PBS rinses and incubation in an avidin–horseradish peroxidase complex (Vector Laboratories, Burlingame, California) following manufacturer's instructions. Conjugates were visualized using a 4-chloro-1-naphthol/peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, Maryland).

Electron microscopic immunocytochemistry

Portions of tentacles were transferred to 4.0% paraformaldehyde/0.5% glutaraldehyde in 0.1 *M* PBS, pH 6.5, for 1 h at room temperature and were transferred to 4.0% paraformaldehyde/0.5% glutaraldehyde in PBS, pH 11, for 8 h at 4°C before washing in PBS, pH 7.4, and dehydration in a graded ethanol series. Samples were infiltrated and embedded in EM-BED-812 (Electron Microscopy Sciences, Ft. Washington, Pennsylvania) according to manufacturer's directions. Silver to gold sections were placed on nickel grids (Ted Pella Inc., Redding, California). Grids were floated on small amounts of reagent at room temperature as follows: (1) 1 h on 0.1 *M* PBS, pH 7.4; (2) 10 min on a saturated solution of sodium metaperiodate, followed by 30 min on several changes of PBS; (3) 1 h on 10% normal serum (Vector Laboratories, Burlingame, California), (4) three successive 30-min rinses on PBS; and (5) 4 h on monoclonal antiserum to synaptophysin (SY38; 1:200). Three successive 30-min rinses on PBS preceded floating the grids on a 1:40 dilution of gold-conjugated IgG (Janssen Life Sciences Products, Piscataway, New Jersey) for 1 h, and two successive 20-min distilled water rinses.

Grids were postfixed for 15 min on a drop of 2% glutaraldehyde and poststained in lead citrate and uranyl acetate, followed by two successive 15-min rinses on distilled water. Specimens were examined and photographed in a JEOL 100CXII electron microscope (JEOL USA, Inc., Peabody, Maine).

Controls consisted of replacement of the primary antiserum with nonimmune serum.

Results

Light microscopic immunocytochemistry

To demonstrate the presence of a synaptophysin-like protein in the tissues of this species, sections of tentacle were examined after application of a monoclonal synaptophysin I antiserum (SY38; Boehringer Mannheim Corp., Indianapolis, Indiana). SY38 has been shown to react with synaptophysin-containing presynaptic vesicles of cerebral and spinal neurons in a variety of species (Wiedenmann and Franke, 1985). The antibody does not recognize synaptophysin II (synaptoporin; Knaus *et al.*, 1990). Figure 1 shows a cross section of tentacle. A fine punctate pattern of immunoreactivity is located in the tissues at the center of the tentacle (Fig. 1A, B, C), an area in and around the mesoglea that is known to contain a dense plexus of nerve cell bodies and processes (Van Marle, 1977; C. DellaCorte, unpub. obs.). Immunolabeling was also assessed using the anti-synaptophysin I antibody C7.2, which recognizes a site in the cytoplasmic tail of the synaptic vesicle protein (Jahn *et al.*, 1985). The extent of immunolabeling and pattern of distribution within the tentacle did not significantly differ from that seen with SY38 (not shown). The staining pattern described was not seen when control IgGs were substituted.

Electron microscopic immunocytochemistry

Labeling was found at the surface of SSVs within the processes of the neural plexus (Fig. 2). Immunogold labeling was exclusive to the SSVs although not all vesicles within the same region showed reaction product. The distribution of the gold particles around SSVs was not homogenous; some regions contained more labeling than others, exhibiting a semi-"corona-like" decoration. Corona decoration of synaptic vesicles has been reported (De Camilli *et al.*, 1983; Wiedenmann and Franke, 1985). No appreciable amount of gold particles was observed over non-neural tissues, although some diffuse background staining was present. As with the sections processed for light microscopy, the specificity of labeling was assessed with controls. Sections adjacent to those immunolabeled with synaptophysin were re-

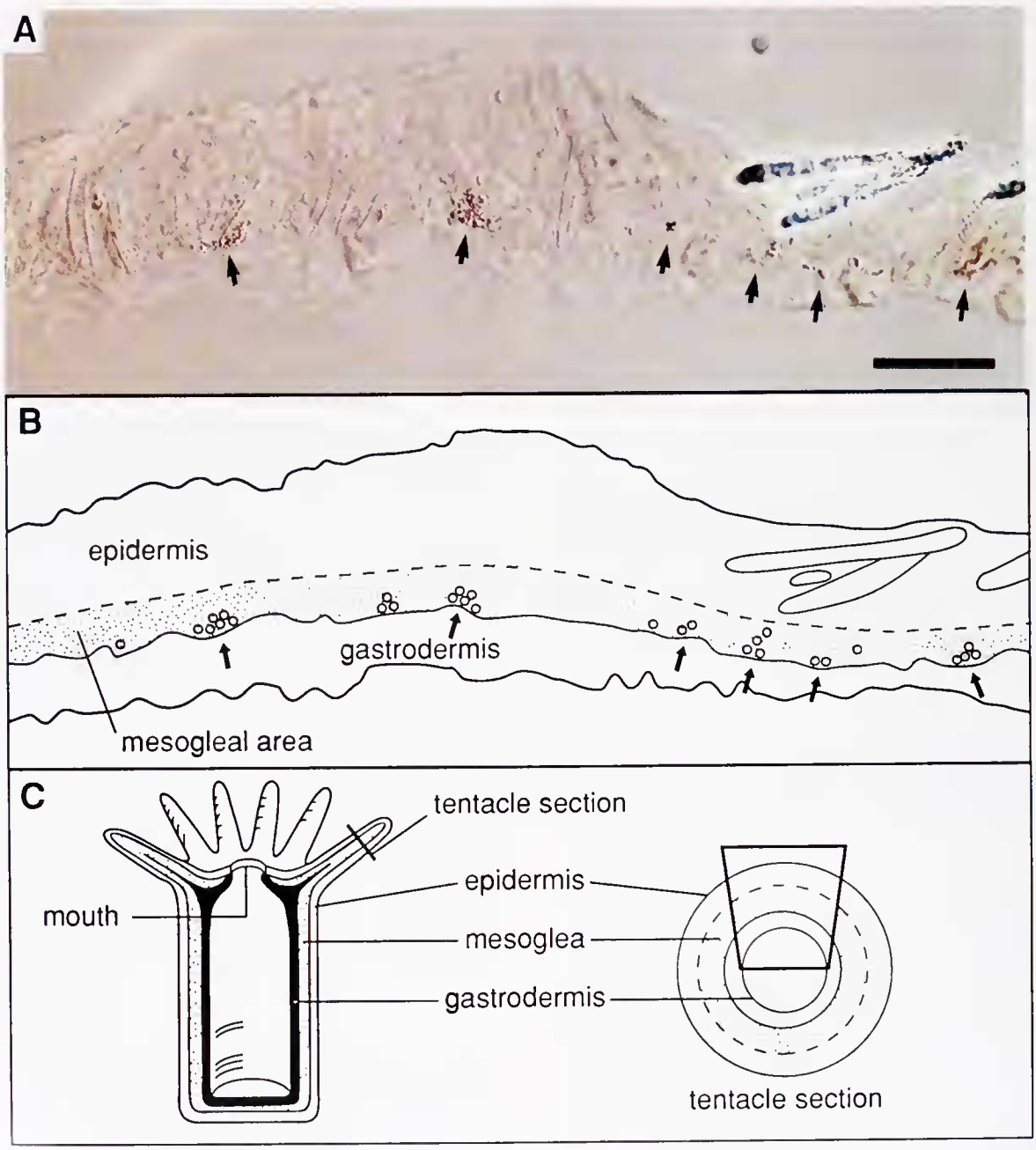

Figure 1. (A) Light microscopy. Cross section of the tentacle of *Condylactis gigantea* immunolabeled with monoclonal anti-synaptophysin (SY38). The epidermal neural plexus consists of a layer, mid-tentacle, between the overlying epidermis and the basal gastrodermis. The section reveals specific, punctate-like labeling within and adjacent to the area of the neural plexus. The scale bar is 20 μm. (B) The epithelial layers of the tentacle are defined in this schematic representation. (C) The arrangement of the tissue within a cross section of tentacle.

acted with nonimmune serum. The labeling described above was absent, or showed only sparse and randomly scattered distribution of gold particles, demonstrating very low background.

Gel electrophoresis and Western blotting

To further demonstrate the presence of synaptophysin-like moieties in this species of anthozoan, we isolated the

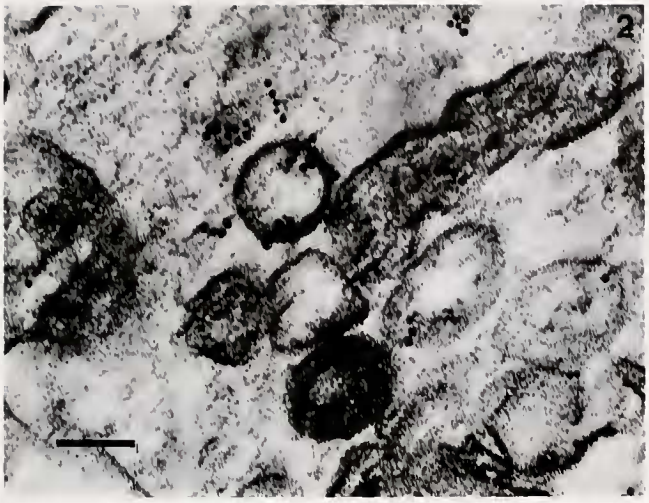

Figure 2. Electron microscopy. Cross section of the tentacle of *Condylactis gigantea* showing immunogold labeling of anti-synaptophysin (SY38). A semi-corona-like reaction product is seen at the surface of a clear-cored synaptic-like vesicle within a neurite in the neural plexus. The scale bar is 0.067 μm.

constituent proteins. When the proteins were visualized with SY38, bands with apparent molecular weights of 78,000 and 114,000 daltons were specifically labeled in both freshly extracted and stored protein fractions (Fig. 3, lanes 4 and 5). The 78,000-dalton protein appeared to predominate after storage, however (Fig. 3, lane 5). The molecular weight of these proteins does not differ considerably from synaptophysin dimers and trimers found in other species (Rehm *et al.*, 1986; Thomas *et al.*, 1988).

In addition, immunoreactivity was assessed with the anti-synaptophysin antibodies G95 (not shown) and C7.2 (Fig. 4). Patterns of synaptophysin-like immunoreactivity at MW_r 78,000 and 114,000 daltons were seen in freshly prepared extracts from *C. gigantea* (lane 4). Similar patterns were also seen in the extracts from rat brain (lane 3). In addition, immunoblotting labeled a 38-kD molecular weight fraction from the extract of *C. gigantea* that had been stored for 1 month at −70°C.

The protein fraction from *C. gigantea* was reacted with a monoclonal antibody to p29, an integral membrane protein present in small clear synaptic vesicles of neurons and endocrine cells. Antibodies to p29 have been shown to cross-react with synaptophysin I (Baumert *et al.*, 1990). Our results show a band with an apparent molecular weight of 29,000 daltons in extracts of tissue from rat brain (Fig. 5). Faint labeling at 38,000 daltons was seen in the protein sample from *C. gigantea* that had been stored (lane 5). A more robust banding pattern was found at 78,000 and 114,000 daltons in both the freshly extracted and the stored protein fractions (lanes 4 and 5). Immunolabeling of a 38-kD band in the rat extract was revealed at 1:150 to 1:200 antibody titers (not shown) (Baumert *et al.*, 1990).

Discussion

Many synaptic vesicle proteins, including synaptophysin, exist in multiple isoforms. At present, two forms of synaptophysin have been identified: synaptophysin I (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985) and synaptophysin II/synaptophorin (Knaus *et al.*, 1990; Bixby, 1992). The properties of synaptophysin I, the focus of our study, have been extensively investigated and show that the glycoprotein contains four membrane-spanning domains, with cytoplasmic amino and carboxy termini (Johnston *et al.*, 1989). The highly immunogenic tail region encompasses 10 copies of a tyrosine-rich repeat (Sudhof *et al.*, 1987). A high degree of homology between synaptophysin I and II suggests that the two forms have similar transmembrane organization, although they share little sequence similarity in the region of the carboxyl-terminal tail (Knaus *et al.*, 1990; Bixby, 1992).

Immunocytochemical light microscopy results show that synaptophysin antibodies recognize an antigenic de-

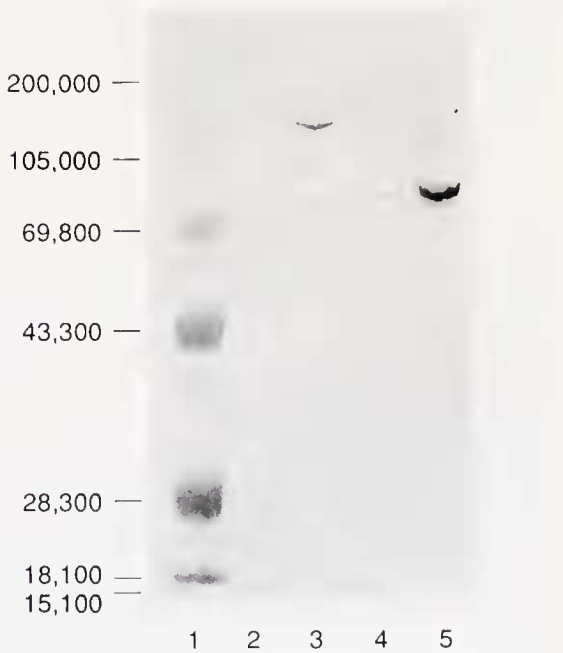

Figure 3. Western blotting. *Condylactis gigantea* synaptophysin-like proteins were visualized using a monoclonal synaptophysin antibody (SY38). Two prominent, high molecular weight protein bands are seen in the membrane fraction of the rat. A faint band is also seen at 38 kD. Bands of MW_r 78 kD and 114 kD were specifically labeled in the protein extract from *C. gigantea*. Lane 1: Molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction, 15 μg/lane; Lane 3: rat brain membrane fraction, 15 μg/lane; Lane 3: freshly prepared fraction of anemone membrane, 15 μg/lane; Lane 4: anemone membrane fraction stored at −70°C for 1 month, 15 μg/lane.

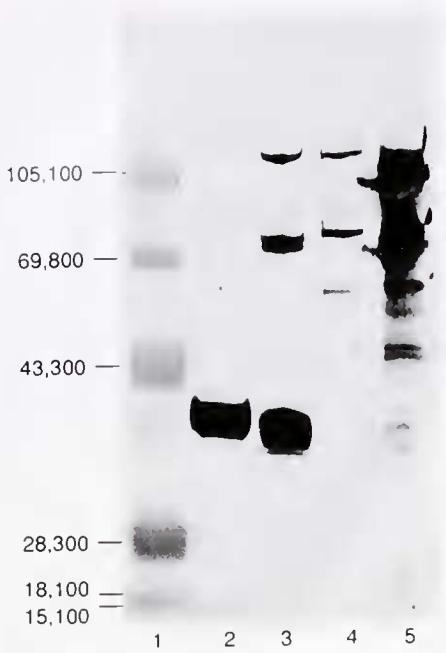

Figure 4. Western blotting. Immunolabeling with antisynaptophysin (C7.2) reveals protein bands at MW_r 78 kD and 114 kD in the freshly made extract from the sea anemone *Condylactis gigantea*, while the stored extract presents another, faint band, at 38 kD. Lane 1: Molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction, 15 μg/lane; Lane 3: rat brain membrane fraction, 15 μg/lane; Lane 4: freshly prepared anemone membrane, 15 μg/lane; Lane 5: anemone membrane fraction stored at −70°C for 1 month, 15 μg/lane.

terminant in the tentacles of *C. gigantea*. In this anthozoan, the epidermal neural plexus forms a discrete layer that is sandwiched between the overlying surface layer of epithelial cells and the epidermal musculature that abuts the mesoglea (C. DellaCorte, unpublished results). As such, it is located almost in the center of the tentacle wall. The positioning of the immunostained product co-localizes with the neural plexus. Electron microscopy shows some antigenic surfaces to be small, clear-cored vesicles within the processes of the neural plexus. Large, dense-cored vesicles did not appear to be labeled. The pattern of labeling (*i.e.*, the overall punctate appearance in light microscopy and the semi-corona-like pattern seen at the surface of putative synaptic vesicles in electron microscopy) are in agreement with those seen in other studies (Bock and Helle, 1977; Navone *et al.*, 1986; Obata *et al.*, 1986; Alder *et al.*, 1992).

Although the synaptophysin molecule has a deduced molecular weight of 38,000 daltons, it has been suggested that the 76,000-dalton dimeric structure may be the native form (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Navone *et al.*, 1986; Rehm *et al.*, 1986). Higher molecular weight homo-multimer complexes are known to form (Wiedenmann and Franke, 1985; Rehm *et al.*, 1986; Jahn *et al.*, 1987; Thomas *et al.*, 1988; Johnston and Sudhof, 1990). Dimers, trimers, and tetramers have been identified following chemical cross-linking studies (Rehm *et al.*, 1986; Thomas *et al.*, 1988; Fykse *et al.*, 1993). Disulfide cross-links also form spontaneously in nontreated extracts (Johnston and Sudhof, 1990; Fykse *et al.*, 1993) and upon storage (Rehm *et al.*, 1986; Thomas *et al.*, 1988). The apparent molecular weights of the synaptophysin-like proteins isolated from the tissues of the sea anemone *Condylactis gigantea* are within the parameters defined by these studies.

The variation in immunoblotting patterns between the SY38 and C7.2 antibodies in our study was initially surprising. Yet similar patterns have been noted. Studies by Wiedemann and Franke (1985) and Rehm *et al.* (1986) reported a lack of synaptophysin immunoreactivity in endocrine cells and a phylogenetic distribution restricted to mammals. Navone and co-workers (1986), however, suggested that these results may have resulted from a limited epitope specificity of SY 38. Their research, using several anti-synaptophysin antibodies including C7.2,

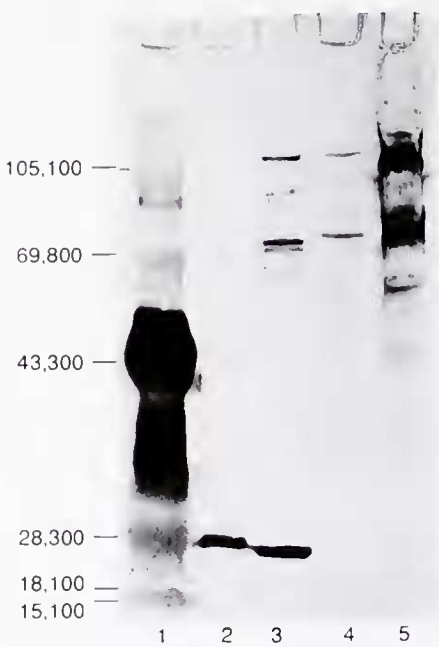

Figure 5. Western blotting. The putative synaptophysin-containing fraction from *Condylactis gigantea* was immunoblotted with monoclonal antibodies to p29. A band with an apparent molecular weight of 29 kD is seen in extracts of tissue from rat brain. In the extract from *C. gigantea* tissues, faint cross-reactive immunolabeling is seen at 38 kD, with more robust banding patterns at 78 kD and 114,000 kD. Lane 1: Molecular weight standards (MW_r); Lane 2: rat brain cytosolic fraction, 15 μg/lane; Lane 3: rat brain membrane fraction, 15 μg/lane; Lane 4: freshly prepared anemone membrane, 15 μg/lane; Lane 5: anemone membrane fraction stored at −70°C for 1 month, 15 μg/lane.

showed the presence of synaptophysin immunolocalization in many vertebrate classes down to amphibia, although not all species were found to display reactivity to every antibody. Furthermore, synaptophysin labeling was found in a variety of neuroendocrine cells.

The protein-containing extract from *C. gigantea* was further characterized by immunoblotting with antibodies directed against p29. The immunogenic tail of synaptophysin is a characteristic shared by the synaptic vesicle protein p29, an integral nonglycosylated protein. Studies demonstrate that this structural relationship between p29 and synaptophysin may result in patterns of antibody cross-reactivity (Baumert *et al.*, 1990) such as that seen in this study with *C. gigantea*.

The data from our study suggest that synaptophysin-like proteins with molecular weights similar to those found in mammalian neural and endocrine systems exist in the sea anemone *C. gigantea*. It will be enlightening to determine the sequence and structure of these proteins and compare them with those from other studies. Comparisons may provide insight into the functional constraints of protein sequences during evolution. Furthermore, characterization of the synaptophysin-like protein by the application of modern techniques on evolutionarily divergent species such as these may be helpful in identifying functionally significant regions of these important molecules.

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